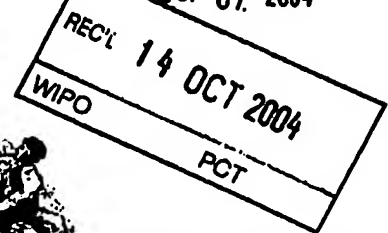PA 1162982

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APPLICATION NUMBER: *60/489,237*
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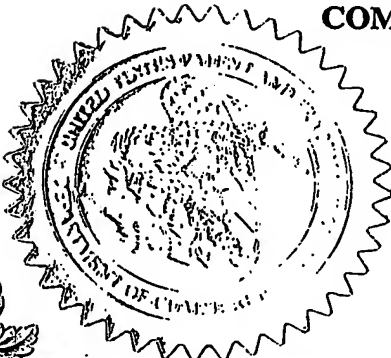
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (c).

DOCKET NUMBER	IT0065PV

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TITLE OF THE INVENTION (500 characters max)

SYNTHETIC GENE ENCODING HUMAN EPIDERMAL GROWTH FACTOR 2/NEU ANTIGEN AND USES THEREOF

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification   *Number of Pages* 38
☒ Drawing(s)   *Number of Sheets* 6
☐ Application Data Sheet. See 37 CFR 1.76

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☐ A check or money order is enclosed to cover the filing fees

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Respectfully submitted,

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EXPRESS MAIL CERTIFICATE

DATE OF DEPOSIT   July 21, 2003
EXPRESS MAIL NO.   EV321984705US

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TITLE OF THE INVENTION
SYNTHETIC GENE ENCODING HUMAN EPIDERMAL GROWTH FACTOR
2/NEU ANTIGEN AND USES THEREOF

5   FIELD OF THE INVENTION
           The present invention relates generally to the therapy of cancer. More
specifically, the present invention relates to synthetic polynucleotides encoding the
human tumor associated polypeptide epidermal growth factor 2/neu antigen, herein
designated hHER2.opt, wherein the polynucleotides are codon-optimized for
10   expression in a human cellular environment. The present invention also provides
recombinant vectors and hosts comprising said synthetic polynucleotides. This
invention also relates to adenoviral vector and plasmid constructs carrying hHER2.opt
and to their use in vaccines and pharmaceutical compositions for preventing and
treating cancer.
15

BACKGROUND OF THE INVENTION
           Epidermal growth factor 2 is a transmembrane tumor associated
antigen, encoded by the HER2/neu proto-oncogene (also called c-erbB-2), which is a
member of the epidermal growth factor receptor family of cell surface receptors. The
20   HER2 gene was originally isolated from a rat neuroglioblastoma (Shih et al., *Nature*
290: 261-264 (1981)) and later cloned and characterized from human cells (Coussens
et al., *Science* 230: 1132-39 (1985); King et al., *Science* 229: 974-76 (1985)).
           HER2/neu is further classified as a member of the HER family of
receptor tyrosine kinases, which consists of four receptors that participate in cell
25   growth and differentiation. The HER receptors contribute to maintaining normal cell
growth by binding growth factor ligands as dimers. Specifically, human HER2 forms
heterodimers with other members of the EGFR family (HER1, HER3 and HER4)
(Klapper et al. *Adv Cancer Res* 77: 25-79 (2000)). Following hHER2 dimerization
and tyrosine auto-phosphorylation, docking sites for cytoplasmic signaling molecules
30   are generated and recruitment of second signaling molecules is initiated. Intracellular
signaling cascades, which ultimately result in the activation of genes important in cell
growth, are thus initiated.
           Low levels of expression of the HER2/neu transcript and the encoded
185 kD protein are normally detected in adult epithelial cells of various tissues,

including the skin and breast, and tissues of the gastrointestinal, reproductive and urinary tracts (Press et al., *Oncogene* 5: 953-962 (1990)). Higher levels of HER2/neu expression are also detected in the corresponding fetal tissues during embryonic development (Press et al., *supra*).

5   Several observations make the HER2 antigen an attractive target for active specific immunotherapy. First, the HER2/neu gene is commonly overexpressed or amplified in various malignancies, such as carcinomas of the breast, ovary, uterus, colon, and prostate, and adenocarcinomas of the lung (reviewed in Disis and Cheever, *Adv. Cancer Research* 71: 343-371 (1997)). Overexpression of

10   HER2/neu correlates with a poor prognosis and a higher relapse rate for cancer patients (Slamon et al., *Science* 244: 707-712 (1989)). Amplification of human HER2 leads to enhanced MAP kinase activity and cell proliferation, and contributes to the aggressive behavior of tumor cells (Ben-Levy et al. *Embo J* 13(14): 3302-11 (1994)). The high expression level of HER2 observed in tumors is in direct contrast with the

15   low levels associated with normal adult tissues.

           Additionally, many cancer patients suffering from malignancies associated with HER2/neu overexpression have had immune responses against the HER2 protein. Anti-hHER2 cytotoxic T lymphocytes (CTL) have been isolated from breast and ovarian cancer patients (Ioannides et al. *Cell Immunol* 151(1): 225-34

20   (1993); Peoples et al. *Proc Natl Acad Sci U S A* 92 (14): 6547-51 (1995)). Several HLA-A2.1-associated hHER2 peptides have been defined and peptide-specific T cells can be generated *in vitro* (Fisk et al. *Cancer Res* 57(1): 8-93 (1997); Yoshino et al. *Cancer Res* 54(13): 3387-90 (1994); Lustgarten et al. *Hum Immunol* 52(2): 109-18 (1997)).

25   The above findings demonstrate that anti-ErbB-2 immune effector mechanisms are activated in cancer patients and highlight the potential benefit of enhancing such immune reactivity. An effective vaccine exploiting the immune response to HER2/neu must both enhance this immunity to a level that is protective and/or preventive and overcome self-tolerance.

30   Based on the above recitation, HER2/neu has been pursued as a target for the development of immunological treatments of malignancies. Anti-HER2 monoclonal antibodies have been investigated as therapies for breast cancer, with each antibody approach demonstrating various levels of success (for discussion, *see* Yarden, *Oncology* 61(suppl 2): 1-13 (2001)). Amici et al. (U.S. Patent No.

6,127,344) disclose a method for inducing immunity against HER2/neu by administering an expression vector comprising the full-length human HER2/neu cDNA functionally linked to the human cytomegalovirus promoter. Cheever and Disis disclose methods for immunizing humans against HER2/neu-associated cancers

5   with HER2 peptides (U.S. Patent No. 5,846,538). Additionally, HER2/neu peptide-based vaccines have been studied in rodent models (*for review, see* Disis and Cheever, *Adv. Cancer Res.* 71:343-71 (1997)).

The development and commercialization of many vaccines have been hindered by difficulties associated with obtaining high expression levels of exogenous

10   genes in successfully transformed host organisms. Therefore, despite the identification of the wild-type nucleotide sequences encoding hHER2 protein described above, it would be highly desirable to develop a readily renewable source of human HER2 protein that utilizes hHER2-encoding nucleotide sequences that are optimized for expression in the intended host cell, said source allowing for the

15   development of a cancer vaccine which is efficacious and not hindered by self-tolerance.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods to elicit or

20   enhance immunity to the protein products expressed by the human HER2 gene, which has been associated with numerous adenocarcinomas, including breast and ovarian cancers. Specifically, the present invention provides polynucleotides encoding human HER2 protein, wherein said polynucleotides are codon-optimized for high level expression in a human cell. The present invention further provides adenoviral and

25   plasmid-based vectors comprising the synthetic polynucleotides and discloses use of said vectors in immunogenic compositions and vaccines for the prevention and/or treatment of HER2-associated cancer. The polynucleotides described herein are more efficient that wild-type HER2 in eliciting a cellular and humoral immune response against human HER2.

30   The present invention also relates to synthetic nucleic acid molecules (polynucleotides) comprising a sequence of nucleotides that encode human epidermal growth factor 2 antigen (hereinafter hHER2) as set forth in SEQ ID NO:2, wherein the synthetic nucleic acid molecules are codon-optimized for high-level expression in a human cell (hereinafter hHER2.opt). The nucleic acid molecules disclosed herein

may be transfected into a host cell of choice wherein the recombinant host cell provides a source for substantial levels of an expressed functional hHER2 protein (SEQ ID NO:2).

The present invention further relates to a synthetic nucleic acid molecule which encodes mRNA that expresses a human HER2 protein; this DNA molecule comprising the nucleotide sequence disclosed herein as SEQ ID NO:1. A preferred aspect of this portion of the present invention is disclosed in FIGURE 1, which shows a DNA molecule (SEQ ID NO:1) that encodes a hHER2 protein (SEQ ID NO:2). The preferred nucleic acid molecule of the present invention is codon-optimized for high-level expression in a human cell. The sequence of this preferred polynucleotide also contains a mutation abolishing tyrosine kinase activity (AAA2257GCC, K753A). Nucleotide sequences that do not contain this mutation are also contemplated by this invention.

The present invention also relates to recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification.

The present invention further relates to a process for expressing a codon-optimized human HER2 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a synthetic polynucleotide encoding a human HER2 protein into a suitable host cell, wherein the synthetic polynucleotide is codon-optimized for optimal expression in a human cell; and, (b) culturing the host cell under conditions which allow expression of said human HER2 protein.

Another aspect of this invention is a method of preventing or treating cancer comprising administering to a mammal a vaccine vector comprising a synthetic nucleic acid molecule, the synthetic nucleic acid molecule comprising a sequence of nucleotides that encodes a human epidermal growth factor 2 antigen (hHER2) protein as set forth in SEQ ID NO:2, wherein the synthetic nucleic acid molecule is codon-optimized for high level expression in a human cell.

The present invention further relates to an adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising: (a) a codon-optimized polynucleotide encoding a human HER2 protein; and (b) a promoter operably linked to the polynucleotide.

The present invention also relates to a vaccine plasmid comprising a plasmid portion and an expression cassette portion, the expression cassette portion comprising: (a) a synthetic polynucleotide encoding a human HER2 protein, wherein the synthetic polynucleotide is codon-optimized for optimal expression in a human cell; and (b) a promoter operably linked to the polynucleotide.

Another aspect of the present invention is a method of protecting a mammal from cancer or treating a mammal suffering from HER2-associated cancer comprising: (a) introducing into the mammal a first vector comprising: i) a codon-optimized polynucleotide encoding a human HER2 protein; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a codon-optimized polynucleotide encoding a human HER2 protein; and ii) a promoter operably linked to the polynucleotide.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

As used throughout the specification and appended claims, the following definitions and abbreviations apply:

The term "promoter" refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers".

The term "cassette" refers to the sequence of the present invention that contains the nucleic acid sequence which is to be expressed. The cassette is similar in concept to a cassette tape; each cassette has its own sequence. Thus by interchanging the cassette, the vector will express a different sequence. Because of the restriction sites at the 5' and 3' ends, the cassette can be easily inserted, removed or replaced with another cassette.

The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus), bacteriophages and cosmids.

The term "first generation," as used in reference to adenoviral vectors, describes said adenoviral vectors that are replication-defective. First generation

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adenovirus vectors typically have a deleted or inactivated E1 gene region, and preferably have a deleted or inactivated E3 gene region.

The designation "pV1J-hHER2.opt" refers to a plasmid construct, disclosed herein, comprising the human CMV immediate-early (IE) promoter with intron A, a full-length codon-optimized human HER2 gene, bovine growth hormone-derived polyadenylation and transcriptional termination sequences, and a minimal pUC backbone (see EXAMPLE 2). The designation "pV1J-hHER2.wt" refers to a construct as described above, except the construct comprises a wild-type human HER2 gene instead of a codon-optimized human HER2 gene.

The designations "MRKAd5-hHER2.opt" and "MRKAd5-hHER2.wt" refer to two constructs, disclosed herein, which comprise an Ad5 adenoviral genome deleted of the E1 and E3 regions. In the "MRKAd5-hHER2.opt" construct, the E1 region is replaced by a codon-optimized human HER2 gene in an E1 parallel orientation under the control of a human CMV promoter without intron A, followed by a bovine growth hormone polyadenylation signal. The "MRKAd5-hHER2.wt" construct is essentially as described above, except the E1 region of the Ad5 genome is replaced with a wild-type human HER2 sequence (see EXAMPLE 3).

The term "effective amount" means sufficient vaccine composition is introduced to produce the adequate levels of the polypeptide, so that an immune response results. One skilled in the art recognizes that this level may vary.

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

"hHER2.wt" and "hHER2.opt" refer to a human epidermal growth factor 2 antigen and a human codon-optimized epidermal growth factor 2 antigen, respectively.

The term "mammalian" refers to any mammal, including a human being.

The abbreviation "Ag" refers to an antigen.

The abbreviations "Ab" and "mAb" refer to an antibody and a monoclonal antibody, respectively.

The abbreviation "ORF" refers to the open reading frame of a gene.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the nucleotide sequence of a codon-optimized polynucleotide (hHER2.opt, SEQ ID NO:1) that encodes human HER2 protein. Panel B shows the deduced amino acid sequence of the human HER2 protein (SEQ ID NO:2). *See* EXAMPLE 1.

FIGURE 2 shows the identification of immunodominant T-cell epitopes in the human HER2 protein by ELISPOT and intracellular staining (ICS) analysis. BALB/c mice immunized with Ad5-hHER2 were analyzed for the induction of human HER2-specific cellular immunity. The number of IFN-γ -secreting anti-human HER2 T cells was determined by ELISPOT on splenocytes from groups of mice (indicated in the first column) using pools or single peptides. Data displayed are representative of several independent experiments. Values are expressed as the number of spot forming colonies (SFC)/10^6 total splenocytes, subtracted of the background values determined in the absence of peptides (typically less than 10 SFC/10^6 total splenocytes). Numbers corresponding to more than three times the background measured in control experiments without antigenic peptides were considered positive values and are indicated in boldface. Frequency of CD4$^+$ or CD8$^+$ T-cell secreting IFN-γ was measured by ICS. Data displayed are representative of several independent experiments. Values are expressed as 1000x[(IFN-γ CD3$^+$ and CD4$^+$ or CD8$^+$)/(CD3$^+$ and CD4$^+$ or CD8$^+$)]. Values higher than 1 % were considered positive and indicated in boldface. Sequences encompassed by the pool or by the single peptide used in the assay are indicated on the left. Numbers refers to the position of amino acid residue of the human HER2 protein.

FIGURE 3 shows the *in vitro* expression of hHER2 following transfection in (A) human embryonic kidney HEK-293 cells (B) and mouse myoblasts C2C7. Data are expressed as the geometric mean of the channel fluorescence from which the signal generated by the empty pV1JnsA plasmid has been subtracted. For C2C7 cells, data are normalized on the efficiency of pEGFP DNA transfection.

FIGURE 4 shows the immune response to human HER2 in BALB/c mice. Panel (A) shows that codon-optimized HER2 yielded significantly improved ELISPOT values compared to wild type HER2. Shown are results from immunization of four groups, each comprising two mice, with plasmid pV1J-

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hHER2.wt or pV1J-hHER2.opt (50μg/dose electroinjected in the quadriceps muscle). Two weeks after the last injection, the frequency of IFN-γ secreting T cells in mouse splenocytes was determined via IFN-γ ELISPOT assay using peptides hNeu15.3 (aa 63-71, including a CD8+ epitope), hNeu301 (aa 1202-1214, including a CD8+

5 epitope) and hNeu42 (aa 165-179, including a CD4+ epitope). Results from 2.5×10^5 and 5×10^5 splenocytes, with two replicas of each amount tested, are shown. Average values were calculated by subtracting the background level determined in the absence of peptides (typically less than 10 SFC/10^6 total splenocytes). Results were expressed as the number of SFC/10^6 total splenocytes. Panel (B) shows that pV1J-

10 hHER2.opt elicits a significantly improved IgG1 and IgG2a humoral response compared to pV1J-hHER2.wt. Serum samples were collected at week 6 (the day before the first immunization, pre-bleed) and week 14 (two weeks after the last injection) from groups of 4 mice immunized with pV1J-hHER2.wt or pV1J-hHER2.opt plasmid DNA. Anti-hHER2 antibody titers in the pooled sera from each

15 group of mice were measured by ELISA using the dimeric extra-cellular domain of hHER2 (HER2-ECD) as target antigen. AP-conjugated goat anti-mouse IgG1 or IgG2a was used to detect bound mouse antibodies.

FIGURE 5 shows a comparison of immunization regimens. Wild-type BALB/c mice and BALB/c transgenic mice overexpressing rat HER2 (indicated as

20 NeuT, see Lucchini et al., *Cancer Lett* 64(3): 203-9 (1992)) were immunized at 6 and 9 weeks of age, either with pV1J-hHER2.wt DNA (50μg/dose, injected in the quadriceps muscle), followed by electrical stimulation or with Ad5-hHER2.wt. At 12 weeks of age, the number of IFN-γ -secreting anti-human cells was determined by ELISPOT analysis from pools of mice using the peptides indicated. Data displayed

25 are representative of several independent experiments. Values are expressed as in FIGURE 1.

FIGURE 6 shows the *in vitro* expression of hHER2 following infection at different m.o.i. in murine liver NMuLi cells. Cells were infected with MRKAd5-hHER2.wt or MRKAd5-hHER2.opt. using various multiplicity of infection (m.o.i.).

30 Values shown are the geometric mean of triplicate determinations. *See* EXAMPLE 7b.

DETAILED DESCRIPTION OF THE INVENTION

Human epidermal growth factor 2 (hHER2) is commonly associated with a number of tumors, including breast, ovarian, gastric, and colon carcinomas. The present invention relates to compositions and methods to elicit or enhance
5 immunity to the protein product expressed by the hHER2 gene, wherein aberrant hHER2 expression is associated with the carcinoma or its development. Association of aberrant hHER2 expression with a carcinoma does not require that the hHER2 protein be expressed in tumor tissue at all time points of its development, as abnormal hHER2 expression may be present at tumor initiation and not be detectable late into
10 tumor progression or vice-versa.

To this end, synthetic DNA molecules encoding the human HER2 protein are provided. The codons of the synthetic molecules are designed so as to use the codons preferred by the projected host cell, which in preferred embodiments is a human cell. The synthetic molecules may be used for the development of plasmid-
15 based vaccines or recombinant adenovirus, which provide effective immunoprophylaxis against HER2-associated cancer through neutralizing antibody and cell-mediated immunity. The synthetic molecules may be used as an immunogenic composition. This invention provides polynucleotides which, when directly introduced into a vertebrate *in vivo*, including mammals such as primates and
20 humans, induce the expression of encoded proteins within the animal.

The wild-type human HER2 nucleotide sequence has been reported. The present invention provides synthetic DNA molecules encoding the human HER2 protein. The synthetic molecules of the present invention comprise a sequence of nucleotides, wherein at least one of the nucleotides has been altered so as to use the
25 codons preferred by a human cell, thus allowing for high-level expression of hHER2 in a human host cell. The synthetic molecules may be used as a source of hHER2 protein, which may be used in a cancer vaccine to provide effective immunoprophylaxis against hHER2-associated carcinomas through neutralizing antibody and cell-mediated immunity.
30 A "triplet" codon of four possible nucleotide bases can exist in over 60 variant forms. Because these codons provide the message for only 20 different amino acids (as well as transcription initiation and termination), some amino acids can be coded for by more than one codon—a phenomenon known as codon redundancy. For reasons not completely understood, alternative codons are not uniformly present in the

endogenous DNA of differing types of cells. Indeed, there appears to exist a variable natural hierarchy or "preference" for certain codons in specific types of cells. As one example, the amino acid leucine is specified by any of six DNA codons including CTA, CTC, CTG, CTT, TTA, and TTG. Exhaustive analysis of genome codon
5   frequencies for microorganisms has revealed endogenous DNA of *E. coli* most commonly contains the CTG leucine-specifying codon, while the DNA of yeasts and slime molds most commonly includes a TTA leucine-specifying codon. In view of this hierarchy, it is generally believed that the likelihood of obtaining high levels of expression of a leucine-rich polypeptide by an *E. coli* host will depend to some extent
10   on the frequency of codon use. For example, it is likely that a gene rich in TTA codons will be poorly expressed in *E. coli*, whereas a CTG rich gene will probably be highly expressed in this host. Similarly, a preferred codon for expression of a leucine-rich polypeptide in yeast host cells would be TTA.

The implications of codon preference phenomena on recombinant
15   DNA techniques are manifest, and the phenomenon may serve to explain many prior failures to achieve high expression levels of exogenous genes in successfully transformed host organisms—a less "preferred" codon may be repeatedly present in the inserted gene and the host cell machinery for expression may not operate as efficiently. This phenomenon suggests that synthetic genes which have been designed
20   to include a projected host cell's preferred codons provide an optimal form of foreign genetic material for practice of recombinant DNA techniques. Thus, one aspect of this invention is a human HER2 gene that is codon-optimized for expression in a human cell. In a preferred embodiment of this invention, it has been found that the use of alternative codons encoding the same protein sequence may remove the
25   constraints on expression of exogenous hHER2 protein in human cells.

In accordance with this invention, the human HER2 gene sequence was converted to a polynucleotide sequence having an identical translated sequence but with alternative codon usage as described by Lathe, "Synthetic Oligonucleotide
30   Probes Deduced from Amino Acid Sequence Data: Theoretical and Practical Considerations" *J. Molec. Biol.* 183:1-12 (1985), which is hereby incorporated by reference. The methodology generally consists of identifying codons in the wild-type sequence that are not commonly associated with highly expressed human genes and replacing them with optimal codons for high expression in human cells. Said optimal

codons are referred to herein as "human-preferred" codons. The new gene sequence is then inspected for undesired sequences generated by codon replacements (e.g., "ATTTA" sequences, inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, high GC content, etc.). Undesirable sequences are

5   eliminated by substitution of the existing codons with different codons coding for the same amino acid. The synthetic gene segments are then tested for improved expression.

The methods described above were used to create synthetic gene sequences for human HER2, resulting in a gene comprising codons optimized for high

10   level expression. While the above procedure provides a summary of our methodology for designing codon-optimized genes for use in cancer vaccines, it is understood by one skilled in the art that similar vaccine efficacy or increased expression of genes may be achieved by minor variations in the procedure or by minor variations in the sequence. One of skill in the art will also recognize that additional DNA molecules

15   may be constructed that provide for high levels of hHER2 expression in human cells, wherein only a portion of the codons of the DNA molecules are codon-optimized.

Accordingly, the present invention relates to a synthetic polynucleotide comprising a sequence of nucleotides encoding a human HER2 protein (SEQ ID NO:2), or a biologically active fragment or mutant form of a human HER2 protein,

20   the polynucleotide sequence comprising codons optimized for expression in a human host. Said mutant forms of the hHER2 protein include, but are not limited to: conservative amino acid substitutions, amino-terminal truncations, carboxy-terminal truncations, deletions, or additions. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially

25   mimics the immunological properties of the hHER2 protein as set forth in SEQ ID NO:2. The synthetic polynucleotides of the present invention encode mRNA molecules that express a functional human HER2 protein so as to be useful in the development of a therapeutic or prophylactic cancer vaccine.

30   The present invention relates to a synthetic nucleic acid molecule (polynucleotide) comprising a sequence of nucleotides which encodes mRNA that expresses a novel hHER2 protein as set forth in SEQ ID NO:2, wherein the synthetic nucleic acid molecule is codon-optimized for high-level expression in a human host

cell. The nucleic acid molecules of the present invention are substantially free from other nucleic acids.

The present invention also relates to recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification. The synthetic DNA molecules, associated vectors, and hosts of the present invention are useful for the development of a cancer vaccine.

A preferred DNA molecule of the present invention comprises the nucleotide sequence disclosed herein as SEQ ID NO:1 (shown in FIGURE 1), which encodes the human HER2 protein shown in FIGURE 2 and set forth as SEQ ID NO:2. The nucleotide sequence set forth in SEQ ID NO:1 was codon-optimized for optimal expression in human cells. To avoid PCR amplification problems, in this embodiment of the present invention, a less stringent optimization design was adopted for the hHER2 sequence between position 3601 and 3805, which reduced GC content while preserving the same amino acid composition. *See* EXAMPLE 5.

One of skill in the art will realize that other HER2 sequences may be designed which are codon-optimized for high-level expression in a human cell, provided that one or more codons are altered to human-preferred codons. It is preferred that at least about 80% of the codons comprising the synthetic HER2 nucleotide sequences of the present invention are human-preferred codons. It is more preferred that at least about 85% of the codons are human-preferred and even more preferred that at least about 90% of the codons are human-preferred.

The present invention also includes biologically active fragments or mutants of SEQ ID NO:1, which encode mRNA expressing human HER2 proteins. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the pharmacological properties of the hHER2 protein, including but not limited to the hHER2 protein as set forth in SEQ ID NO:2. Any such polynucleotide includes but is not necessarily limited to: nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations. The mutations of the present invention encode mRNA molecules that express a functional hHER2 protein in a eukaryotic cell so as to be useful in cancer vaccine development.

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This invention also relates to synthetic codon-optimized DNA molecules that encode the hHER2 protein, wherein the nucleotide sequence of the synthetic DNA differs significantly from the nucleotide sequence of SEQ ID NO:1, but still encodes the hHER2 protein as set forth in SEQ ID NO:2. Such synthetic DNAs are intended to be within the scope of the present invention. Therefore, the present invention discloses codon redundancy that may result in numerous DNA molecules expressing an identical protein. Also included within the scope of this invention are mutations in the DNA sequence that do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in the functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide that has properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or receptor for a ligand.

The present invention also relates to hHER2opt fusion constructs, including but not limited to fusion constructs which express a portion of the human HER2 protein linked to various markers, including but in no way limited to GFP (Green fluorescent protein), the MYC epitope, GST, and Fc. Any such fusion construct may be expressed in the cell line of interest and used to screen for modulators of the human HER2 protein disclosed herein. Also contemplated are fusion constructs that are constructed to enhance the immune response to human HER2 including, but not limited to: DOM, hsp70, and LTB.

The present invention further relates to recombinant vectors that comprise the synthetic nucleic acid molecules disclosed throughout this specification. These vectors may be comprised of DNA or RNA. For most cloning purposes, DNA vectors are preferred. Typical vectors include plasmids, modified viruses, Baculorvirus, bacteriophage, cosmids, yeast artificial chromosomes, and other forms of episomal or integrated DNA that can encode a hHER2 protein. It is well within the

purview of the skilled artisan to determine an appropriate vector for a particular gene transfer or other use.

An expression vector containing codon-optimized DNA encoding a hHER2 protein may be used for high-level expression of hHER2 in a recombinant

5 host cell. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Also, a variety of bacterial expression vectors may be used to express recombinant hHER2 in bacterial cells if desired. In addition, a variety of fungal cell expression vectors may be used to express recombinant hHER2 in fungal cells. Further, a variety of insect cell

10 expression vectors may be used to express recombinant protein in insect cells.

The present invention also relates to host cells transformed or transfected with vectors comprising the nucleic acid molecules of the present invention. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells

15 including, but not limited to, cell lines of bovine, porcine, monkey and rodent origin; and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Such recombinant host cells can be cultured under suitable conditions to produce hHER2 or a biologically equivalent form. In a preferred embodiment of the present invention, the host cell is human. As defined herein, the term "host cell" is

20 not intended to include a host cell in the body of a transgenic human being, transgenic human fetus, 'or transgenic human embryos.

As noted above, an expression vector containing DNA encoding a hHER2 protein may be used for expression of hHER2 in a recombinant host cell.

25 Therefore, another aspect of this invention is a process for expressing a human HER2 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a codon-optimized nucleic acid that encodes human HER2 into a suitable human host cell; and, (b) culturing the host cell under conditions which allow expression of said human HER2 protein.

30 A preferred embodiment of this aspect of this invention provides a process for expressing a human HER2 protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid as set forth in SEQ ID NO:1 into a suitable human host cell; and, (b) culturing the host cell under conditions which allow expression of said human HER2 protein.

Following expression of hHER2 in a host cell, hHER2 protein may be recovered to provide hHER2 protein in active form. Several hHER2 protein purification procedures are available and suitable for use. Recombinant hHER2

5   protein may be purified from cell lysates and extracts by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography. In addition, recombinant hHER2 protein can be separated from other cellular proteins by use of an immunoaffinity column

10   made with monoclonal or polyclonal antibodies specific for full-length hHER2 protein, or polypeptide fragments of hHER2 protein.

The nucleic acids of the present invention may be assembled into an expression cassette which comprises sequences designed to provide for efficient

15   expression of the protein in a human cell. The cassette preferably contains a full-length codon-optimized hHER2 gene, with related transcriptional and translations control sequences operatively linked to it, such as a promoter, and termination sequences. In a preferred embodiment, the promoter is the cytomegalovirus promoter without the intron A sequence (CMV), although those skilled in the art will recognize

20   that any of a number of other known promoters such as the strong immunoglobulin, or other eukaryotic gene promoters may be used. A preferred transcriptional terminator is the bovine growth hormone terminator, although other known transcriptional terminators may also be used. The combination of CMV-BGH terminator is particularly preferred.

25   In accordance with this invention, the hHER2opt expression cassette is inserted into a vector. The vector is preferably an adenoviral vector, although linear DNA linked to a promoter, or other vectors, such as adeno-associated virus or a modified vaccinia virus, retroviral or lentiviral vector may also be used.

30   If the vector chosen is an adenovirus, it is preferred that the vector be a so-called first-generation adenoviral vector. These adenoviral vectors are characterized by having a non-functional E1 gene region, and preferably a deleted adenoviral E1 gene region. In some embodiments, the expression cassette is inserted in the position where the adenoviral E1 gene is normally located. In addition, these

vectors optionally have a non-functional or deleted E3 region. It is preferred that the adenovirus genome used be deleted of both the E1 and E3 regions (ΔE1ΔE3). The adenoviruses can be multiplied in known cell lines which express the viral E1 gene, such as 293 cells, or PERC.6 cells, or in cell lines derived from 293 or PERC.6 cell

5   which are transiently or stabilely transformed to express an extra protein. For examples, when using constructs that have a controlled gene expression, such as a tetracycline regulatable promoter system, the cell line may express components involved in the regulatory system. One example of such a cell line is T-Rex-293; others are known in the art.

10   For convenience in manipulating the adenoviral vector, the adenovirus may be in a shuttle plasmid form. This invention is also directed to a shuttle plasmid vector which comprises a plasmid portion and an adenovirus portion, the adenovirus portion comprising an adenoviral genome which has a deleted E1 and optional E3 deletion, and has an inserted expression cassette comprising codon-optimized human

15   HER2. In preferred embodiments, there is a restriction site flanking the adenoviral portion of the plasmid so that the adenoviral vector can easily be removed. The shuttle plasmid may be replicated in prokaryotic cells or eukaryotic cells.

        In a preferred embodiment of the invention, the expression cassette is inserted into the pMRKAd5-HV0 adenovirus plasmid (*See* Emini et al., WO

20   02/22080, which is hereby incorporated by reference). This plasmid comprises an Ad5 adenoviral genome deleted of the E1 and E3 regions. The design of the pMRKAd5-HV0 plasmid was improved over prior adenovectors by extending the 5' cis-acting packaging region further into the E1 gene to incorporate elements found to be important in optimizing viral packaging, resulting in enhanced virus amplification.

25   Advantageously, this enhanced adenoviral vector is capable of maintaining genetic stability following high passage propagation.

        Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the adenoviruses, shuttle plasmids, and DNA immunogens of this invention.

30

        It has been determined in accordance with the present invention that the synthetic cDNA molecule described herein (SEQ ID NO:1), which is codon-optimized for high-level expression in a human cell, is expressed with greater efficiency than the corresponding wild type sequence. The codon optimized cDNA of

hHER2 breaks tolerance to hHER2 more efficiently than the wild type sequence. Additionally, it was shown herein that hHER2opt is more immunogenic that hHER2 and is more efficient in eliciting both cellular and humoral immune responses.

5   Therefore, the vectors described above may be used in immunogenic compositions and vaccines for preventing the development of adenocarcinomas associated with aberrant HER2 expression and/or for treating existing cancers. The vectors of the present invention allow for vaccine development and commercialization by eliminating difficulties with obtaining high expression levels of exogenous HER2
10   in successfully transformed host organisms. To this end, one aspect of the instant invention is a method of preventing or treating cancer comprising administering to a mammal a vaccine vector comprising a synthetic codon-optimized nucleic acid molecule, the synthetic codon-optimized nucleic acid molecule comprising a sequence of nucleotides that encodes a human HER2 protein as set forth in SEQ ID NO:2.
15   In accordance with the method described above, the vaccine vector may be administered for the treatment or prevention of cancer in any mammal. In a preferred embodiment of the invention, the mammal is a human.

          Further, one of skill in the art may choose any type of vector for use in the treatment and prevention method described. Preferably, the vector is an
20   adenovirus vector or a plasmid vector. In a preferred embodiment of the invention, the vector is an adenoviral vector comprising an adenoviral genome with a deletion in the adenovirus E1 region, and an insert in the adenovirus E1 region, wherein the insert comprises an expression cassette comprising: (a) a synthetic codon-optimized polynucleotide encoding a human HER2 protein; and (b) a promoter operably linked
25   to the polynucleotide.

          The instant invention further relates to an adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising: (a) a synthetic codon-optimized polynucleotide encoding a human HER2 protein; and (b) a
30   promoter operably linked to the polynucleotide.

          In a preferred embodiment of this aspect of the invention, the adenovirus vector is an Ad 5 vector.

          In other preferred embodiments of the invention, the vector is an Ad6 vector or an Ad24 vector.

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In another aspect, the invention relates to a vaccine plasmid comprising a plasmid portion and an expression cassette portion, the expression cassette portion comprising: (a) a synthetic codon-optimized polynucleotide encoding a human HER2 protein; and (b) a promoter operably linked to the polynucleotide.

5

In some embodiments of this invention, the recombinant adenovirus vaccines disclosed herein are used in various prime/boost combinations with a plasmid-based polynucleotide vaccine in order to induce an enhanced immune response. In this case, the two vectors are administered in a "prime and boost"
10 regimen. For example the first type of vector is administered, then after a predetermined amount of time, for example, 2 weeks, 1 month, 2 months, six months, or other appropriate interval, a second type of vector is administered. Preferably the vectors carry expression cassettes encoding the same polynucleotide or combination of polynucleotides. In the embodiment where a plasmid DNA is also used, it is
15 preferred that the vector contain one or more promoters recognized by mammalian or insect cells. In a preferred embodiment, the plasmid would contain a strong promoter such as, but not limited to, the CMV promoter. The synthetic human HER2 gene or other gene to be expressed would be linked to such a promoter. An example of such a plasmid would be the mammalian expression plasmid V1Jns as described (J. Shiver
20 *et. al.* in *DNA Vaccines*, M. Liu et al. eds., N.Y. Acad. Sci., N.Y., 772:198-208 (1996), which is herein incorporated by reference).

As stated above, an adenoviral vector vaccine and a plasmid vaccine may be administered to a vertebrate as part of a single therapeutic regime to induce an immune response. To this end, the present invention relates to a method of protecting
25 a mammal from cancer comprising: (a) introducing into the mammal a first vector comprising: i) a synthetic codon-optimized polynucleotide encoding a human HER2 protein; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a synthetic codon-optimized polynucleotide encoding a human
30 HER2 protein; and ii) a promoter operably linked to the polynucleotide.

In one embodiment of the method of protection described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid.

The instant invention further relates to a method of treating a mammal suffering from an adenocarcinoma comprising: (a) introducing into the mammal a first vector comprising: i) a synthetic codon-optimized polynucleotide encoding a human HER2 protein; and ii) a promoter operably linked to the polynucleotide; (b)

5   allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a synthetic codon-optimized polynucleotide encoding a human HER2 protein; and ii) a promoter operably linked to the polynucleotide.

10   In one embodiment of the method of treatment described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid.

The amount of expressible DNA or transcribed RNA to be introduced
15   into a vaccine recipient will depend partially on the strength of the promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10 μg to 300 μg of a plasmid vaccine vector is administered directly into muscle tissue. An effective dose for recombinant adenovirus is approximately
20     $10^6 - 10^{12}$ particles and preferably about $10^7 - 10^{11}$ particles. Subcutaneous injection, intradermal introduction, impression though the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations may be provided. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other
25   means of administration with adjuvants such as interleukin 12 protein, concurrently with or subsequent to parenteral introduction of the vaccine of this invention is also advantageous.

The vaccine vectors of this invention may be naked, i.e., unassociated with any proteins, adjuvants or other agents which impact on the recipient's immune
30   system. In this case, it is desirable for the vaccine vectors to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, it may be advantageous to administer an immunostimulant, such as an adjuvant, cytokine, protein, or other carrier with the vaccines or immunogenic compositions of the present invention. Therefore, this invention includes the use of

such immunostimulants in conjunction with the compositions and methods of the present invention. An immunostimulant, as used herein, refers to essentially any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. Said immunostimulants can be administered in the form of DNA or protein. Any of a variety of immunostimulants may be employed in conjunction with the vaccines and immunogenic compositions of the present inventions, including, but not limited to: GM-CSF, IFNα, tetanus toxoid, IL12, B7.1, LFA-3 and ICAM-1. Said immunostimulants are well-known in the art. Agents which assist in the cellular uptake of DNA, such as, but not limited to calcium ion, may also be used. These agents are generally referred to as transfection facilitating reagents and pharmaceutically acceptable carriers. Those of skill in the art will be able to determine the particular immunostimulant or pharmaceutically acceptable carrier as well as the appropriate time and mode of administration.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

EXAMPLE 1

Human HER2 optimized codon sequence.

The entire hHER2.opt coding sequence was synthesized and assembled by BIONEXUS (Bionexus Inc. Oakland CA.) and cloned into pCR-blunt vector (Invitrogen, The Netherlands). The hHER2.opt cDNA was constructed using oligonucleotides assembled by PCR. For many experiments described herein, the

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hHER2.opt nucleotide sequence used carried an optimized Kozak sequence at its 5'-end, the complete nucleotide sequence as set forth below:

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   1 GCCGCCACCA TGGAGCTGGC CGCCCTGTGC CGCTGGGGCC TGCTGCTGGC
  51 CCTGCTGCCC CCCGGCGCCG CCAGCACCCA GGTGTGCACC GGCACCGACA
 101 TGAAGCTGCG CCTGCCCGCC AGCCCCGAGA CCCACCTGGA CATGCTGCGC
 151 CACCTGTACC AGGGCTGCCA GGTGGTGCAG GGCAACCTGG AGCTGACCTA
 201 CCTGCCCACC AACGCCAGCC TGAGCTTCCT GCAGGACATC CAGGAGGTGC
 251 AGGGCTACGT GCTGATCGCC CACAACCAGG TGCGCCAGGT GCCCCTGCAG
 301 CGCCTGCGCA TCGTGCGCGG CACCCAGCTG TTCGAGGACA ACTACGCCCT
 351 GGCCGTGCTG GACAACGGCG ACCCCCTGAA CAACACCACC CCCGTGACCG
 401 GCGCCAGCCC CGGCGGCCTG CGCGAGCTGC AGCTGCGCAG CCTGACCGAG
 451 ATCCTGAAGG GCGGCGTGCT GATCCAGCGC AACCCCCAGC TGTGCTACCA
 501 GGACACCATC CTGTGGAAGG ACATCTTCCA CAAGAACAAC CAGCTGGCCC
 551 TGACCCTGAT CGACACCAAC CGCAGCCGCG CCTGCCACCC CTGCAGCCCC
 601 ATGTGCAAGG CAGCCGCTG CTGGGGCGAG AGCAGCGAGG ACTGCCAGAG
 651 CCTGACCCGC ACCGTGTGCG CCGGCGGCTG CGCCCGCTGC AAGGGCCCCC
 701 TGCCCACCGA CTGCTGCCAC GAGCAGTGCG CCGCCGGCTG CACCGGCCCC
 751 AAGCACAGCG ACTGCCTGGC CTGCCTGCAC TTCAACCACA GCGGCATCTG
 801 CGAGCTGCAC TGCCCCGCCC TGGTGACCTA CAACACCGAC ACCTTCGAGA
 851 GCATGCCCAA CCCCGAGGGC CGCTACACCT TCGGCGCCAG CTGCGTGACC
 901 GCCTGCCCCT ACAACTACCT GAGCACCGAC GTGGGCAGCT GCACCCTGGT
 951 GTGCCCCCTG CACAACCAGG AGGTGACCGC CGAGGACGGC ACCCAGCGCT
1001 GCGAGAAGTG CAGCAAGCCC TGCGCCCGCG TGTGCTACGG CCTGGGCATG
1051 GAGCACCTGC GCGAGGTGCG CGCCGTGACC AGCGCCAACA TCCAGGAGTT
1101 CGCCGGCTGC AAGAAGATCT TCGGCAGCCT GGCCTTCCTG CCCGAGAGCT
1151 TCGACGGCGA CCCCGCCAGC AACACCGCCC CCCTGCAGCC CGAGCAGCTG
1201 CAGGTGTTCG AGACCCTGGA GGAGATCACC GGCTACCTGT ACATCAGCGC
1251 CTGGCCCGAC AGCCTGCCCG ACCTGAGCGT GTTCCAGAAC CTGCAGGTGA
1301 TCCGCGGCCG CATCCTGCAC AACGGCGCCT ACAGCCTGAC CCTGCAGGGC
1351 CTGGGCATCA GCTGGCTGGG CCTGCGCAGC CTGCGCGAGC TGGGCAGCGG
1401 CCTGGCCCTG ATCCACCACA ACACCCACCT GTGCTTCGTG CACACCGTGC
1451 CCTGGGACCA GCTGTTCCGC AACCCCCACC AGGCCCTGCT GCACACCGCC
1501 AACCGCCCCG AGGACGAGTG CGTGGGCGAG GGCCTGGCCT GCCACCAGCT
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1551 GTGCGCCCGC GGCCACTGCT GGGGCCCCGG CCCCACCCAG TGCGTGAACT
1601 GCAGCCAGTT CCTGCGCGGC CAGGAGTGCG TGGAGGAGTG CCGCGTGCTG
1651 CAGGGCCTGC CCCGCGAGTA CGTGAACGCC CGCCACTGCC TGCCCTGCCA
1701 CCCCGAGTGC CAGCCCCAGA ACGGCAGCGT GACCTGCTTC GGCCCCGAGG
1751 CCGACCAGTG CGTGGCCTGC GCCCACTACA AGGACCCCCC CTTCTGCGTG
1801 GCCCGCTGCC CCAGCGGCGT GAAGCCCGAC CTGAGCTACA TGCCCATCTG
1851 GAAGTTCCCC GACGAGGAGG CGCCTGCCA GCCCTGCCCC ATCAACTGCA
1901 CCCACAGCTG CGTGGACCTG GACGACAAGG GCTGCCCCGC CGAGCAGCGC
1951 GCCAGCCCCC TGACCAGCAT CATCAGCGCC GTGGTGGGCA TCCTGCTGGT
2001 GGTGGTGCTG GGCGTGGTGT TCGGCATCCT GATCAAGCGC CGCCAGCAGA
2051 AGATCCGCAA GTACACCATG CGCCGCCTGC TGCAGGAGAC CGAGCTGGTG
2101 GAGCCCCTGA CCCCCAGCGG CGCCATGCCC AACCAGGCCC AGATGCGCAT
2151 CCTGAAGGAG ACCGAGCTGC GCAAGGTGAA GGTGCTGGGC AGCGGCGCCT
2201 TCGGCACCGT GTACAAGGGC ATCTGGATCC CCGACGGCGA GAACGTGAAG
2251 ATCCCCGTGG CCATCGCCGT GCTGCGCGAG AACACCAGCC CCAAGGCCAA
2301 CAAGGAGATC CTGGACGAGG CCTACGTGAT GGCCGGCGTG GGCAGCCCCT
2351 ACGTGAGCCG CCTGCTGGGC ATCTGCCTGA CCAGCACCGT GCAGCTGGTG
2401 ACCCAGCTGA TGCCCTACGG CTGCCTGCTG GACCACGTGC GCGAGAACCG
2451 CGGCCGCCTG GGCAGCCAGG ACCTGCTGAA CTGGTGCATG CAGATCGCCA
2501 AGGGCATGAG CTACCTGGAG GACGTGCGCC TGGTGCACCG CGACCTGGCC
2551 GCCCGCAACG TGCTGGTGAA GAGCCCCAAC CACGTGAAGA TCACCGACTT
2601 CGGCCTGGCC CGCCTGCTGG ACATCGACGA GACCGAGTAC CACGCCGACG
2651 GCGGCAAGGT GCCCATCAAG TGGATGGCCC TGGAGAGCAT CCTGCGCCGC
2701 CGCTTCACCC ACCAGAGCGA CGTGTGGAGC TACGGCGTGA CCGTGTGGGA
2751 GCTGATGACC TTCGGCGCCA AGCCCTACGA CGGCATCCCC GCCCGCGAGA
2801 TCCCCGACCT GCTGGAGAAG GGCGAGCGCC TGCCCCAGCC CCCCATCTGC
2851 ACCATCGACG TGTACATGAT CATGGTGAAG TGCTGGATGA TCGACAGCGA
2901 GTGCCGCCCC CGCTTCCGCG AGCTGGTGAG CGAGTTCAGC CGCATGGCCC
2951 GCGACCCCCA GCGCTTCGTG GTGATCCAGA ACGAGGACCT GGGCCCCGCC
3001 AGCCCCCTGG ACAGCACCTT CTACCGCAGC CTGCTGGAGG ACGACGACAT
3051 GGGCGACCTG GTGGACGCCG AGGAGTACCT GGTGCCCCAG CAGGGCTTCT
3101 TCTGCCCCGA CCCCGCCCCC GGCGCCGGCG GCATGGTGCA CCACCGCCAC
3151 CGCAGCAGCA GCACCCGCAG CGGCGGCGGC GACCTGACCC TGGGCCTGGA
3201 GCCCAGCGAG GAGGAGGCCC CCCGCAGCCC CCTGGCCCCC AGCGAGGGCG
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3251 CCGGCAGCGA CGTGTTCGAC GGCGACCTGG GCATGGGCGC CGCCAAGGGC
3301 CTGCAGAGCC TGCCCACCCA CGACCCCAGC CCCCTGCAGC GCTACAGCGA
3351 GGACCCCACC GTGCCCCTGC CCAGCGAGAC CGACGGCTAC GTGGCCCCCC
3401 TGACCTGCAG CCCCCAGCCC GAGTACGTGA ACCAGCCCGA CGTGCGCCCC
3451 CAGCCCCCCA GCCCCGCGA GGGCCCCCTG CCCGCCGCCC GCCCCGCCGG
3501 CGCCACCCTG GAGCGCCCCA AGACCCTGAG CCCCGGCAAG AACGGCGTGG
3551 TGAAGGACGT GTTCGCCTTC GGCGGCGCCG TGGAGAACCC CGAGTACCTG
3601 ACCCCCCAGG GCGGAGCTGC TCCTCAGCCT CACCCTCCAC CTGCTTTCAG
3651 CCCTGCTTTC GACAACCTGT ACTACTGGGA CCAGGACCCT CCTGAGAGGG
3701 GTGCTCCTCC TAGCACCTTC AAGGGCACCC CCACCGCCGA GAACCCCGAG
3751 TACCTGGGCC TGGACGTGCC CGTGTAAA (SEQ ID NO: 9)
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In addition, the ATP binding Lysine residue 753 was substituted with Alanine (K753A) by replacing codon AAA with GCA. This mutation abrogates tyrosine kinase activity of the corresponding protein and eliminates the downstream signaling events and the oncogenic activity of either human (Messerle et al. *Mol Cell Endocrinol* 105(1): 1-10 (1994)) or rat (Ben Levi et al., *supra*). In addition, the kinase-deficient K756A mutant can inactivate the signaling activity of a co-expressed oncogenic hHER2.wt.

EXAMPLE 2

Plasmid Constructs

pV1J-hHER2.wt: Human HER2 wild type coding sequence was amplified by PCR from plasmid pLTR-2/erb-B2 (kindly provided by P. Di Fiore, European Institute of Oncology, Milan, Italy; (Di Fiore et al. *Science* 237 (4811): 178-82 (1987)) using primers hNeu.for1 (5'-C C A G T T T A A A C A T T T A A A T G C C G C C A C C A T G G A G C T G G C G G C C T -3'; (SEQ ID NO:3 coding sequence is underlined) and hNeu.rev2 (5'- G C C G T C G A C T T T A C A C T G G C A C G T C C A G A C C C A -3' (SEQ ID NO:4) and TaKaRa LA Taq polymerase (TaKaRa Otsu, Shiga, Japan). The amplification product, which incorporates an optimized translation start site (Kozak, M., *J Mol Biol* 196(4): 947-50 (1987); Kozak, M., *Nucleic Acids Res* 15(20): 8125-48 (1987)), was digested with

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*Pme*I and *Sal*I restriction enzymes and cloned into the *Eco*RV and *Sal*I sites of mammalian expression plasmid pV1JnsA (Montgomery et al. *DNA Cell Biol* 12(9): 777-83 (1993)). The plasmid pV1J-hHER2 thus generated contained the full-length wild type human HER2 sequence under the transcriptional control of the human cytomegalovirus immediate-early promoter with its intron A sequence. The human wild type HER2 coding sequence was followed by the bovine growth hormone polyadenylation signal sequence.

5

pV1J-hHER2.opt: Plasmid pCR-hHER2opt was digested with *Eco*RI for 1 hr at 37°C. The resulting 2156 bp insert was purified and cloned into the *Eco*RI site of plasmid pV1JnsB (Montgomery et al., *supra*).

10

EXAMPLE 3

Adenovirus Vectors

MRKAd5-hHER2.wt: A *Swa*I-*Sal*I DNA fragment from pV1j_hHER2 containing the human HER2 cDNA was cloned in the corresponding sites of the shuttle plasmid polyMRKΔE1 (Bett et al., *Proc Natl Acad Sci U S A* 91(19): 8802-04 (1994)). The resulting plasmid pMRKΔE1_hHER2 contained a human CMV promoter driving the expression of the human HER2 cDNA, followed by the bovine growth hormone polyadenylation signal. Plasmid pMRKΔE1_hHER2 was recombined with the adenoviral backbone plasmid pAd5_HV0 to generate the pre-adenoviral plasmid pAd5-hHER2.

15

20

MRKAd5-hHER2.opt: Plasmid pCR-hHER2opt was digested with *Eco*RI. The resulting 2156 bp insert was purified and cloned into the *Eco*RI of the polyMRK-Ad5 shuttle plasmid (*See* Emini et al., WO 02/22080, which is hereby incorporated by reference). Plasmids pAd5-humHER2.wt and pMRKAd5-hHER2.opt were linearized by *Pac*I digestion and transfected into PerC6 cells to generate Ad5-hHER2 recombinant adenovirus. The viruses were grown in large quantities by multiple rounds of amplification and purified by caesium chloride gradient ultra-centrifugation (Fallaux et al., *Hum Gene Ther* 9(13): 1909-17 (1998)). Viral DNA was extracted by proteinase K digestion and genomic integrity was confirmed by restriction analysis.

25

30

EXAMPLE 4

<u>Mice immunization.</u>

Six-week old inbred female BALB/c mice (H-2^d; kindly provided by
G. Forni, University of Turin) were kept in standard conditions. Mice were treated in
5   accordance with European union and institutional guidelines. In particular, mice were
fully anesthetized with ketamine (Imalgene 500; Merial Italia, Milano, Italy) at 100
mg/kg of body weight and xylazine (Xilor, BIO 98; S. Lazzaro, Bologna, Italy) at 5.2
mg/kg when necessary for procedures.

Fifty micrograms of plasmid DNA were electroinjected in a 50 µl
10   volume in mice quadriceps at 6, 8, and 10 weeks of age, as previously described
(Rizzuto et al. *Proc. Natl. Acad. Sci. U.S.A.* 96(11): 6417-22 (1999)). 50µg of
pCMV_hNeu optimized or not were injected without incising the skin into both
quadriceps muscles (25 µg in 50µl of physiologic solution/injection) and
electrostimulation (ES) was performed as previously described (Zucchelli et al. *J.*
15   *Virol.* 74(24): 11598-607 (2000); Rizzuto et al., *supra*). Briefly, electrical shock
consisted of 10 trains with 1000 bipolar pulses (130V, 75mA, 200µs/phase).

Ad injections were carried out in mice quadriceps in 50 µl volumes.
Sera were collected at 7 wks (1wk before first immunization, pre-bleed), and 12 wks
(two weeks after the last immunization).
20

EXAMPLE 5

<u>Codon optimized cDNA of hHER2 significantly increased hHER2 expression.</u>

A synthetic human HER2 gene (hHER2.opt, FIGURE 1) was designed
to incorporate human-preferred (humanized) codons for each amino acid (hereinafter
25   aa) residue. During assembly of the gene, PCR amplification consistently deleted an
86 bp sequence starting from position 3642, due to the high GC content of the
sequence in this region. To overcome this problem, a less stringent optimization
design was adopted for the hHER2 sequence between position 3601 and 3805, which
reduced GC content while preserving the same aa composition.

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The codon optimized cDNA was modified to maintain 83.9% nucleotide identity to the original clone. The codon optimized cDNAs were cloned into the pV1J vectors (Montgomery et al., *supra*), placing in front a Kozak optimized sequence (5'-GCCGCCACC-3', SEQ ID NO:8) and under the control of the human

5   cytomegalovirus (CMV)/intron A promoter plus the bovine growth hormone (BGH) termination signal. The construct was named pV1J-hHER2opt (see EXAMPLE 2).

     In vitro transfection of HEK-293 or C2.7 cells showed that the codon optimized sequence dramatically improves hHER2 expression compared to the wt sequence (FIGURE 3).

10   An adenovirus type 5 vector was constructed carrying the hHER2opt sequence flanked by the CMV/intron A promoter and the BGH termination signal (Ad5-hHER2.opt). For comparison, the equivalent plasmid and Ad5 vectors were constructed carrying the wild type hHER2 sequence yielding pV1J-hHER2.wt and Ad5-hHER2.wt. Similar to those containing the codon optimized cDNA, these

15   vectors carry the wild type gene under the control of the CMV/int A promoter with BGH termination signal.

     Infection of NMuLi cells at various m.o.i demonstrated an improved expression of the codon-optimized sequence compared to the wt sequence.

20   EXAMPLE 6

<u>Mouse IFN-γ ELISPOT Assay.</u>

     Mouse splenocytes secreting IFN-γ in an antigen-specific manner were detected using a standard enzyme-linked immunospot (ELISPOT) assay (Miyahira et al. *J Immunol Methods* 181(1): 45-54 (1995)). Multiscreen 96-well MAIP filtration

25   plates (cat. No. MAIPS4510; Millipore, Bedford, MA) were coated with an affinity-purified rat anti-mouse IFN-γ antibody ((IgG1, clone R4-6A2, cat No. 18181D, Pharmingen, San Diego, CA) diluted sterile PBS. After overnight incubation, plates were washed with PBST (0.005% Tween in PBS) and incubated with R10 medium for 2 hrs at 37°C to block non-specific binding.

30   Splenocytes were obtained by removing the spleen from the euthanized mice in a sterile manner. Spleen disruption was carried out by grating the dissected spleen on a metal grid. Red blood cells were removed by osmotic lysis by adding 1

ml of 0.1X PBS to the cell pellet and vortexing no more than 15 seconds. One ml of 2X PBS was then added and the volume was brought to 4 ml with 1X PBS. Cells were pelleted by centrifugation at 1200 rpm for 10 min at room temp., and the pellet was resuspended in 1 ml R10 medium (RPMI 1640 supplemented with 10% fetal calf

5   serum, 2 mM L-glutamine, 50 U of penicillin per ml, 50 µg of streptomycin per ml, 10 mM HEPES, 50 µM 2-mercaptoethanol). Viable cells were counted using Türks staining.

Splenocytes derived from the spleen of two or more immunized mice were incubated for 15 hrs in the presence of 6 µg/ml of a single or a pool of peptides,

10   at a density of $2.5 - 5 \ 10^5$ cells/well. Concanavalin A (ConA) was used as positive internal control for each mouse at 5 µg/ml. After extensive washing with PBST, biotinylated rat anti-mouse IFN-gamma antibody (cat No. 18112D, PharMingen; San Diego, CA) was added. The plates were incubated at 4°C overnight and then washed with PBST prior to the addition of streptavidin-alkaline phosphatase (Cat No.

15   13043E, PharMingen; San Diego, CA). After incubation for 2 hrs at room temperature the plates were extensively washed with PBST and developed by incubating with a one-step nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate substrate (cat. No. 34042, Pierce, Rockford, IL) for 5 to 15 min for development of spots. Rinsing the plates in water stopped the reaction. DMSO and Concanavalin A (10 µg/ml) were

20   included as background and positive control for each sample. Spots were counted by computer-assisted imaging analysis (AID ELR02 coupled with AID ELISPOT 2.6 Software, Strassberg, Germany).

The frequency of positive IFN-γ producing splenocytes per total number §of cells plated per well was calculated as the average value of spots derived

25   from duplicates at two different cell concentrations subtracted of the average value similarly derived from spots measured in control wells containing non-pulsed splenocytes. Changes in frequency of IFN-γ producing cells were defined as exceeding a 95% confidence bound calculated from measurements of controls. Differences with a p value < 0.05 were considered significant.

30

EXAMPLE 7

a) *In vitro* expression of plasmid constructs.

pV1J-hHER2.wt and pV1J.hHER2.opt *in vitro* expression was assessed by transiently transfecting human embryonic kidney HEK-293 or mouse

5   myoblasts C2C7 cell lines and detecting human HER2 expression by flow cytometry. Supercoiled, endotoxin-free plasmid DNA pVIJ-hHER2-wt encoding the human HER2 expression cassette used for immunization was purified from *E. coli* DH12S cells (Invitrogen, Groningen, The Netherlands) by Qiagen endo-free plasmid Giga Kit (Qiagen, Hilden, Germany).

10   Plasmids pVIJ-hHER2.wt or pVIJ-hHER2.opt were lipofectamine-transfected (Gibco BRL Invitrogen, Groningen, The Netherlands) in HEK-293 cells. Similarly, mouse myoblasts C2C7 cells were transfected with a 1:1 or 10:1 mixture of plasmid pHygEGFP (BD Biosciences Clontech, PaloAlto, CA) and pV1J-hHER2.wt or pV1J-hHER2.opt.

15

b) *In vitro* expression of adenovirus vectors.

Murine liver NMuli cells were infected with MRKAd5-hHER2.wt or MRKAd5-hHER2.opt. using various multiplicity of infection (m.o.i.). *See* FIGURE 6. Confluent NMuLi cells were infected with CsCl-purified adenovirus for 30 min at

20   RT. Unbound virus was removed, cells washed and incubated in fresh medium for 48hrs for protein expression. Cell extract and luciferase assays were performed with the Luciferase Assay System (Promega, Madison, WI), according to manufacturer instructions. Luciferase activity is expressed as relative light units (RLU) per mg of protein.

25   After 48 hrs of incubation for protein expression, cells were collected using trypsin and stained by a standard indirect immunofluorescence procedure using mAb Ab-2 anti hHER2 or mAb Ab-5 anti-hHER2 (Oncogene, Boston, MA) as primary antibody, followed by RPE-conjugated goat anti-mouse IgG1 and anti mouse IgG (Cat.no. Star81PE and STAR76, Diamedix, Miami, FL), respectively. All

30   samples were acquired within 24 hrs of staining using a FACSCalibur flow cytometer and CellQuest software (BD Immunocytometry Systems, San Diego, CA).

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EXAMPLE 8

Identification of immunodominant T-cell epitopes in the human HER2 protein

Three hundred and twelve 15-amino acid peptides, overlapping by 11
5   amino acids, were designed to span the entire human HER2 sequence. These
peptides, which also included seven peptides designed to overcome insolubility
problems, were synthesized by SynPep (Dublin, CA). All peptides were shown to be
> 90% pure by HPLC and were used without HPLC purification. Peptides were
reconstituted at 35 mg/ml in DMSO. Those peptides that did not immediately
10   dissolve were rocked at 37°C to aid dissolution. If necessary, 1 to 3 additional
volume(s) of DMSO were added to fully dissolve those peptides that were still not in
solution after several hours of rocking. Reconstituted peptides were combined so that
each peptide was equally represented in the mix. The final concentration of each
peptide in the mix was calculated to be 1 mg/ml. Each mix was aliquoted and stored
15   at -80°C.

To identify the immunodominant T-cell epitopes of the human HER2
gene in BALB/c mice (H-2^d genetic background), 6-week-old female BALB/c mice
were immunized by injecting 10^9 vp of Ad5-hHER2 in the quadriceps muscles. A
second injection was performed after 3 weeks. A second group of mice was similarly
20   injected with saline solution as negative control. Three weeks after the second
injection, the animals were sacrificed and the frequency of IFN-γ secreting T cells in
mouse splenocytes was evaluated by interferon-γ enzyme-linked immunospot (IFN-γ
ELISPOT) assay.

Three hundred and eleven peptides, each 15 amino acids long,
25   overlapping by 11 residues, and spanning the entire human HER2 protein sequence
were combined into eleven pools indicated with alphabetical letters from A to K, from
N- to C-terminus. Each of these pools was tested for its ability to stimulate IFN-γ
spleen T cells. For peptide pools A, B and M, IFN-γ ELISPOT measured a
statistically significant IFN-γ production by mice immunized with Ad5-hHER2 as
30   compared to control in the absence of peptide. To identify the individual peptide
responsible for the activity, peptides from pool A, B and M were divided into sub-
pools, among which A$_{III}$ and A$_{IV}$, B$_{III}$ and M$_I$ scored positive. Single peptides from

these positive subpools were then tested for their ability to trigger IFN-γ release. Overlapping peptides hNeu-15 and hNeu-16 exhibited high and comparable reactivity. A much lower reactivity was exhibited by overlapping peptides hNeu-41 and hNeu-42. Another peptide, hNeu-301, was also shown to contain a T-cell

5 epitope.

To confirm these data and identify the CD4+ or CD8+ T cell subset responsible for IFN-γ production, IFN-γ secreting T-cells were characterized by intracellular staining (ICS). Mouse splenocytes were incubated with single peptides for 12 hrs in the presence of the secretion inhibitor brefeldin A, fixed, permeabilized

10 and then stained for intracellular IFN-γ, CD3 and CD4 or CD8 markers and analyzed by flow cytometry. ICS confirmed the reactivity of peptide hNeu-15, identifying it as an epitope able to activate CD8+ cells. Peptide hNeu15 and hNeu16 were equally reactive in ELISPOT analysis, suggesting that the CD8+ epitope should be comprised in the 11aa residues common to the two peptides. To identify the target nonamer

15 sequence, we tested three 9 aa long peptides spanning the overlapping region between hNeu15 and hNeu16. hNeu15.3 proved the most reactive, displaying a slightly increased reactivity compared to the 15 aa long peptides hNeu-15 and hNeu-16. Interestingly, about half of the reactivity was also detected with hNeu15.1, indicating that two overlapping but distinct CD8+ epitopes co-exist in this 11 aa sequence.

20 IFN-γ ICS analysis also confirmed the reactivity of hNeu301 and typed it as a CD8+ epitope. Analysis of these CD8+ epitopes by IFN-γ ELISPOT confirmed the results obtained by ICS. Finally, a low reactivity was detected for hNeu41 and hNeu42 peptides, whose low response was predominantly CD4+.

25   EXAMPLE 9

Intracellular Cytokine Staining.

Intracellular IFN-γ production was measured according to BD Pharmingen standard protocol. Briefly, 2×10^6 spleen cells were cultured for 15 hrs in R10 medium in the presence of 6 µg/ml of single or pool of peptides and Brefeldin

30 A as protein transport inhibitor (Cytofix/Cytoperm Plus™ with GolgiPlug™ Kit; BD Pharmingen; San Diego, CA). Staphylococcus Enterotoxin B (SEB) at 10 µg/ml (cat.

No. S4881, SIGMA, Saint Louis, MI) and DMSO were tested with the splenocytes as positive and background control, respectively.

Before staining of surface antigens, Ab anti-mouse CD16/CD32 was used to reduce non-specific immunofluorescent signal (cat No. 553142, BD
5   PharMingen, San Diego, CA). The specific signal was obtained with APC-anti-mouse CD3e, PE-anti-mouse CD4 and PerCP-anti-mouse CD8a (cat. No. 553066, 553653 and 553036, BD Pharmingen; San Diego, CA). The cells were then washed, fixed, permeabilized and stained for intracellular IFN-γ using FITC-conjugated mAb (cat. No.554411,BD Pharmingen San Diego, CA). T lymphocyte IFN-γ was
10   calculated as 1000 x [(IFN-γ^+, CD3$^+$ and CD4$^+$ or CD8$^+$)/(CD3$^+$ andCD4$^+$ or CD8$^+$)]. Generally, at least 50,000 CD3$^+$ lymphocytes were collected by simultaneously gating on CD3$^+$ events and small lymphocytes. All samples were acquired within 24 hrs of staining using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA).

15

EXAMPLE 10

Antibody Titration and Isotyping.

Sera for antibody titration were obtained by retro-orbital bleeding. ELISA plates (Nunc Maxisorp™, Roskilde, Denmark) were coated overnight at 4°C
20   with goat anti-human IgG Fc-specific (Pierce; Cat.n. 31123) at a concentration of 2 µg/ml in 50mM NaHCO3 (pH 9.6). Excess of antibody was removed and non-specific binding blocked by incubating for 60min at 37°C in PBBST5 buffer (BSA 5%. Tween 0.05%). After washing, supernatant of IgB2-cells was added in saturating condition and incubated at RT for 2hrs (Chen et al. *J Biol Chem* 271(13): 7620-9
25   (1996)). IgB2, kindly provided by Dr. Y. Yarden, Weizmann Institute of Science, Rehovot, Israel), are HEK-293 cells secreting the dimeric fusion between the extracellular domain of HER2 and the Fc portion of human. Plates were washed and serial dilution of sera (from 1:4,000 to 1:25,600) in PBBST1 buffer (BSA 1%, Tween 0.05%) were incubated overnight at 4°C. Pre-immune sera were used as background.
30   Washes were carried out with PBBST1. Secondary antibody (goat anti-mouse IgG1 or IgG2a AP-conjugated (Pharmingen ,557272 and 553389) was diluted 1:40,000 in PBBST5 and incubated 2-3 hr at room temp. on a shaker. After washing, plates were

developed by incubation with Sigma 106 phosphatase substrate (Sigma; cat.n. A106) in diethanolamine. Plates were read by an automated ELISA reader (Labsistems Multiskan Bichromatic, Helsinki,Finland) and the results were expressed as $A = A_{405nm} - A_{620nm}$. For each sample, the background signal detected with the pre-

5   immune serum was subtracted.

Anti-hHER2 serum titers were calculated as the reciprocal limiting dilution of serum producing an absorbance at least 3-fold greater than the absorbance of autologous pre-immune serum at the same dilution.

10   EXAMPLE 11

Increased Immunogenicity of hHER2opt.

To examine *in.vivo* immune responses induced by the wild type and codon optimized hHER2 expression vectors, BALB/c mice were immunized intramuscularly with m 1×10^9 vp of MRKAd5-hHER2.wt or MRKAd5-hHER2.opt.

15   Mice were subjected to three injections two weeks apart, at 6, 8 and 10 weeks of age. Two weeks after the last immunization, splenocytes were isolated from each mouse. To quantify the IFNγ secreting hHER2-specific CD8 T-cell precursor frequencies generated by the adenovirus-mediated immunization, the ELISPOT assay for the H-2^d restricted T-cell epitope hNeu15.3, hNeu301 and hNeu42 was used. Immunization

20   elicited a measurable immune response yielding 53 IFNγ spot forming cells (SFC, mean value) specific for the tested epitopes. The SFC increased in the group immunized with 1×10^9 vp of MRKAd5-hHER2.opt. No peptide-specific IFN γ SFC were detected in the Ad5 immunized mice (data not shown).

Sera from mice immunized with 1×10^9 vp of each hHER2 adenovirus

25   vector were tested in ELISA using the IgB2 protein as substrate (FIGURE 3B). hHER2-specific antibody titer in MRKAd5-hHER2.opt immunized mice was detected in all immunized mice and the geometric mean value of the Ab titer was 46,474. In contrast, the MRKAd5-hHER2.wt immunized group showed an approximately 100 fold lower geometric mean titer of hHER2-specific antibody. Thus, these results

30   demonstrate that the codon optimized cDNA of hHER2 is more efficient in eliciting a cellular and humoral immune response.

EXAMPLE 12

Statistical Analysis.

Where indicated, results were analyzed by the Student t test. A p value
< 0.05 was considered significant.

5

WHAT IS CLAIMED IS:

1.   A synthetic nucleic acid molecule comprising a sequence of nucleotides that encodes a human HER2/neu protein as set forth in SEQ ID NO:2, the synthetic nucleic acid molecule being codon-optimized for high level expression in a
5   human cell.

2.   The synthetic nucleic acid molecule of claim 1 wherein the nucleic acid is DNA.

10   3.   The synthetic nucleic acid molecule of claim 1 wherein the nucleic acid is mRNA.

4.   The synthetic nucleic acid molecule of claim 1 wherein the nucleic acid is cDNA.
15

5.   The synthetic nucleic acid molecule of claim 1 wherein the sequence of nucleotides comprises the sequence of nucleotides set forth in SEQ ID NO:1.

20   6.   A vector comprising the nucleic acid molecule of claim 1.

7.   A host cell comprising the vector of claim 6.

8.   A process for expressing a human HER2/neu protein in a
25   recombinant host cell, comprising:
             (a)   introducing a vector comprising the nucleic acid of claim 1 into a suitable host cell; and,
             (b)   culturing the host cell under conditions which allow expression of said human HER2 protein.
30

9.   A method of preventing or treating cancer comprising administering to a mammal a vaccine vector comprising a synthetic codon-optimized nucleic acid molecule, the nucleic acid molecule comprising a sequence of nucleotides that encodes a human HER2/neu protein as set forth in SEQ ID NO:2.

10.   A method according to claim 9 wherein the mammal is human.

11.   A method according to claim 9 wherein the vector is an adenovirus vector or a plasmid vector.

12.   A method according to claim 9 wherein the vector is an adenoviral vector comprising an adenoviral genome with a deletion in the adenovirus E1 region, and an insert in the adenovirus E1 region, wherein the insert comprises an expression cassette comprising:

        (a)   a codon-optimized polynucleotide encoding a human HER2 protein; and

        (b)   a promoter operably linked to the polynucleotide.

13.   A method according to claim 9 wherein the vector is a plasmid vaccine vector, which comprises a plasmid portion and an expressible cassette comprising

        (a)   a codon-optimized polynucleotide encoding a human HER2 protein; and

        (b)   a promoter operably linked to the polynucleotide.

14.   An adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising:

        (a)   a codon-optimized polynucleotide encoding a human HER2 protein; and

        (b)   a promoter operably linked to the polynucleotide.

15.   An adenovirus vector according to claim 14 which is an Ad 5 vector.

16.   An adenovirus vector according to claim 14 which is an Ad 6 vector.

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17.   An adenovirus vector according to claim 14 which is an Ad 24 vector.

18.   A vaccine plasmid comprising a plasmid portion and an expression cassette portion, the expression cassette portion comprising:

(a)   a codon-optimized polynucleotide encoding a human HER2 protein; and

(b)   a promoter operably linked to the polynucleotide.

19.   A method of protecting a mammal from cancer comprising:

(a)   introducing into the mammal a first vector comprising:

i)   a codon-optimized polynucleotide encoding a human HER2 protein; and

ii)   a promoter operably linked to the polynucleotide;

(b)   allowing a predetermined amount of time to pass; and

(c)   introducing into the mammal a second vector comprising:

i)   a codon-optimized polynucleotide encoding a human HER2 protein; and

ii)   a promoter operably linked to the polynucleotide.

20.   A method according to claim 19 wherein the first vector is a plasmid and the second vector is an adenovirus vector.

21.   A method according to claim 19 wherein the first vector is an adenovirus vector and the second vector is a plasmid.

22.   A method of treating a mammal suffering from a colorectal carcinoma comprising:

(a)   introducing into the mammal a first vector comprising:

i)   a codon-optimized polynucleotide encoding a human HER2 protein; and

              ii)   a promoter operably linked to the

polynucleotide;

     (b)   allowing a predetermined amount of time to pass; and

     (c)   introducing into the mammal a second vector

5   comprising:

             i)   a codon-optimized polynucleotide encoding a

human HER2 protein; and

             ii)   a promoter operably linked to the

polynucleotide.

10

     23.   A method according to claim 22 wherein the first vector is a plasmid and the second vector is an adenovirus vector.

     24.   A method according to claim 22 wherein the first vector is an

15   adenovirus vector and the second vector is a plasmid.

TITLE OF THE INVENTION
SYNTHETIC GENE ENCODING HUMAN EPIDERMAL GROWTH FACTOR
2/NEU ANTIGEN AND USES THEREOF

5   ABSTRACT OF THE DISCLOSURE

Synthetic polynucleotides encoding human HER2/neu are provided, the synthetic polynucleotides being codon-optimized for expression in a human cellular environment. The gene encoding hHER2 is commonly associated with the development of human carcinomas. The present invention provides compositions and
10   methods to elicit or enhance immunity to the protein product expressed by the hHER2 tumor-associated antigen, wherein aberrant hHER2 expression is associated with a carcinoma or its development. This invention specifically provides adenoviral vector and plasmid constructs carrying codon-optimized human HER2 and discloses their use in vaccines and pharmaceutical compositions for preventing and treating cancer.

15

FIGURE 1. Human Codon-Optimized HER2/neu Nucleotide Sequence

A.

```
   1 ATGGAGCTGG CCGCCCTGTG CCGCTGGGGC CTGCTGCTGG CCCTGCTGCC
  51 CCCCGGCGCC GCCAGCACCC AGGTGTGCAC CGGCACCGAC ATGAAGCTGC
 101 GCCTGCCCGC CAGCCCCGAG ACCCACCTGG ACATGCTGCG CCACCTGTAC
 151 CAGGGCTGCC AGGTGGTGCA GGGCAACCTG GAGCTGACCT ACCTGCCCAC
 201 CAACGCCAGC CTGAGCTTCC TGCAGGACAT CCAGGAGGTG CAGGGCTACG
 251 TGCTGATCGC CCACAACCAG GTGCGCCAGG TGCCCCTGCA GCGCCTGCGC
 301 ATCGTGCGCG GCACCCAGCT GTTCGAGGAC AACTACGCCC TGGCCGTGCT
 351 GGACAACGGC GACCCCCTGA ACAACACCAC CCCCGTGACC GGCGCCAGCC
 401 CCGGCGGCCT GCGCGAGCTG CAGCTGCGCA GCCTGACCGA GATCCTGAAG
 451 GGCGGCGTGC TGATCCAGCG CAACCCCCAG CTGTGCTACC AGGACACCAT
 501 CCTGTGGAAG GACATCTTCC ACAAGAACAA CCAGCTGGCC CTGACCCTGA
 551 TCGACACCAA CCGCAGCCGC GCCTGCCACC CCTGCAGCCC CATGTGCAAG
 601 GGCAGCCGCT GCTGGGGCGA GAGCAGCGAG GACTGCCAGA GCCTGACCCG
 651 CACCGTGTGC GCCGGCGGCT GCGCCCGCTG CAAGGGCCCC CTGCCCACCG
 701 ACTGCTGCCA CGAGCAGTGC GCCGCCGGCT GCACCGGCCC CAAGCACAGC
 751 GACTGCCTGG CCTGCCTGCA CTTCAACCAC AGCGGCATCT GCGAGCTGCA
 801 CTGCCCCGCC CTGGTGACCT ACAACACCGA CACCTTCGAG AGCATGCCCA
 851 ACCCCGAGGG CCGCTACACC TTCGGCGCCA GCTGCGTGAC CGCCTGCCCC
 901 TACAACTACC TGAGCACCGA CGTGGGCAGC TGCACCCTGG TGTGCCCCCT
 951 GCACAACCAG GAGGTGACCG CCGAGGACGG CACCCAGCGC TGCGAGAAGT
1001 GCAGCAAGCC CTGCGCCCGC GTGTGCTACG GCCTGGGCAT GGAGCACCTG
1051 CGCGAGGTGC GCGCCGTGAC CAGCGCCAAC ATCCAGGAGT TCGCCGGCTG
1101 CAAGAAGATC TTCGGCAGCC TGGCCTTCCT GCCCGAGAGC TTCGACGGCG
1151 ACCCCGCCAG CAACACCGCC CCCCTGCAGC CCGAGCAGCT GCAGGTGTTC
1201 GAGACCCTGG AGGAGATCAC CGGCTACCTG TACATCAGCG CCTGGCCCGA
1251 CAGCCTGCCC GACCTGAGCG TGTTCCAGAA CCTGCAGGTG ATCCGCGGCC
1301 GCATCCTGCA CAACGGCGCC TACAGCCTGA CCCTGCAGGG CCTGGGCATC
1351 AGCTGGCTGG GCCTGCGCAG CCTGCGCGAG CTGGGCAGCG GCCTGGCCCT
1401 GATCCACCAC AACACCCACC TGTGCTTCGT GCACACCGTG CCCTGGGACC
1451 AGCTGTTCCG CAACCCCCAC CAGGCCCTGC TGCACACCGC CAACCGCCCC
1501 GAGGACGAGT GCGTGGGCGA GGGCCTGGCC TGCCACCAGC TGTGCGCCCG
1551 CGGCCACTGC TGGGGCCCCG CCCCCACCCA GTGCGTGAAC TGCAGCCAGT
1601 TCCTGCGCGG CCAGGAGTGC GTGGAGGAGT GCCGCGTGCT GCAGGGCCTG
1651 CCCCGCGAGT ACGTGAACGC CCGCCACTGC CTGCCCTGCC ACCCCGAGTG
1701 CCAGCCCCAG AACGGCAGCG TGACCTGCTT CGGCCCCGAG GCCGACCAGT
1751 GCGTGGCCTG CGCCCACTAC AAGGACCCCC CCTTCTGCGT GGCCCGCTGC
1801 CCCAGCGGCG TGAAGCCCGA CCTGAGCTAC ATGCCCATCT GGAAGTTCCC
1851 CGACGAGGAG GGCGCCTGCC AGCCCTGCCC CATCAACTGC ACCCACAGCT
1901 GCGTGGACCT GGACGACAAG GGCTGCCCCG CCGAGCAGCG CGCCAGCCCC
1951 CTGACCAGCA TCATCAGCGC CGTGGTGGGC ATCCTGCTGG TGGTGGTGCT
2001 GGGCGTGGTG TTCGGCATCC TGATCAAGCG CCGCCAGCAG AAGATCCGCA
2051 AGTACACCAT GCGCCGCCTG CTGCAGGAGA CCGAGCTGGT GGAGCCCCTG
2101 ACCCCCAGCG GCGCCATGCC CAACCAGGCC CAGATGCGCA TCCTGAAGGA
2151 GACCGAGCTG CGCAAGGTGA AGGTGCTGGG CAGCGGCGCC TTCGGCACCG
2201 TGTACAAGGG CATCTGGATC CCCGACGGCG AGAACGTGAA GATCCCCGTG
2251 GCCATCGCCG TGCTGCGCGA GAACACCAGC CCCAAGGCCA CAAGGAGAT
2301 CCTGGACGAG GCCTACGTGA TGGCCGGCGT GGGCAGCCCC TACGTGAGCC
2351 GCCTGCTGGG CATCTGCCTG ACCAGCACCG TGCAGCTGGT GACCCAGCTG
2401 ATGCCCTACG GCTGCCTGCT GGACCACGTG CGCGAGAACC GCGGCCGCCT
2451 GGGCAGCCAG GACCTGCTGA ACTGGTGCAT GCAGATCGCC AAGGGCATGA
2501 GCTACCTGGA GGACGTGCGC CTGGTGCACC GCGACCTGGC CGCCCGCAAC
2551 GTGCTGGTGA AGAGCCCCAA CCACGTGAAG ATCACCGACT TCGGCCTGGC
2601 CCGCCTGCTG GACATCGACG AGACCGAGTA CCACGCCGAC GGCGGCAAGG
2651 TGCCCATCAA GTGGATGGCC CTGGAGAGCA TCCTGCGCCG CCGCTTCACC
2701 CACCAGAGCG ACGTGTGGAG CTACGGCGTG ACCGTGTGGG AGCTGATGAC
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2751 CTTCGGCGCC AAGCCCTACG ACGGCATCCC CGCCCGCGAG ATCCCCGACC
2801 TGCTGGAGAA GGGCGAGCGC CTGCCCCAGC CCCCCATCTG CACCATCGAC
2851 GTGTACATGA TCATGGTGAA GTGCTGGATG ATCGACAGCG AGTGCCGCCC
2901 CCGCTTCCGC GAGCTGGTGA GCGAGTTCAG CCGCATGGCC CGCGACCCCC
2951 AGCGCTTCGT GGTGATCCAG AACGAGGACC TGGGCCCCGC CAGCCCCCTG
3001 GACAGCACCT TCTACCGCAG CCTGCTGGAG GACGACGACA TGGGCGACCT
3051 GGTGGACGCC GAGGAGTACC TGGTGCCCCA GCAGGGCTTC TTCTGCCCCG
3101 ACCCCGCCCC CGGCGCCGGC GGCATGGTGC ACCACCGCCA CCGCAGCAGC
3151 AGCACCCGCA GCGGCGGCGG CGACCTGACC CTGGGCCTGG AGCCCAGCGA
3201 GGAGGAGGCC CCCCGCAGCC CCCTGGCCCC CAGCGAGGGC GCCGGCAGCG
3251 ACGTGTTCGA CGGCGACCTG GGCATGGGCG CCGCCAAGGG CCTGCAGAGC
3301 CTGCCCACCC ACGACCCCAG CCCCCTGCAG CGCTACAGCG AGGACCCCAC
3351 CGTGCCCCTG CCCAGCGAGA CCGACGGCTA CGTGGCCCCC CTGACCTGCA
3401 GCCCCCAGCC CGAGTACGTG AACCAGCCCG ACGTGCGCCC CCAGCCCCCC
3451 AGCCCCCGCG AGGGCCCCCT GCCCGCCGCC CGCCCCGCCG GCGCCACCCT
3501 GGAGCGCCCC AAGACCCTGA GCCCCGGCAA GAACGGCGTG GTGAAGGACG
3551 TGTTCGCCTT CGGCGGCGCC GTGGAGAACC CCGAGTACCT GACCCCCCAG
3601 GGCGGAGCTG CTCCTCAGCC TCACCCTCCA CCTGCTTTCA GCCCTGCTTT
3651 CGACAACCTG TACTACTGGG ACCAGGACCC TCCTGAGAGG GGTGCTCCTC
3701 CTAGCACCTT CAAGGGCACC CCCACCGCCG AGAACCCCGA GTACCTGGGC
3751 CTGGACGTGC CCGTGTAA (SEQ ID NO:1)
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B. Human Her2/Neu Protein Sequence

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   1 MELAALCRWG LLLALLPPGA ASTQVCTGTD MKLRLPASPE THLDMLRHLY QGCQVVQGNL
  61 ELTYLPTNAS LSFLQDIQEV QGYVLIAHNQ VRQVPLQRLR IVRGTQLFED NYALAVLDNG
 121 DPLNNTTPVT GASPGGLREL QLRSLTEILK GGVLIQRNPQ LCYQDTILWK DIFHKNNQLA
 181 LTLIDTNRSR ACHPCSPMCK GSRCWGESSE DCQSLTRTVC AGGCARCKGP LPTDCCHEQC
 241 AAGCTGPKHS DCLACLHFNH SGICELHCPA LVTYNTDTFE SMPNPEGRYT FGASCVTACP
 301 YNYLSTDVGS CTLVCPLHNQ EVTAEDGTQR CEKCSKPCAR VCYGLGMEHL REVRAVTSAN
 361 IQEFAGCKKI FGSLAFLPES FDGDPASNTA PLQPEQLQVF ETLEEITGYL YISAWPDSLP
 421 DLSVFQNLQV IRGRILHNGA YSLTLQGLGI SWLGLRSLRE LGSGLALIHH NTHLCFVHTV
 481 PWDQLFRNPH QALLHTANRP EDECVGEGLA CHQLCARGHC WGPGPTQCVN CSQFLRGQEC
 541 VEECRVLQGL PREYVNARHC LPCHPECQPQ NGSVTCFGPE ADQCVACAHY KDPPFCVARC
 601 PSGVKPDLSY MPIWKFPDEE GACQPCPINC THSCVDLDDK GCPAEQRASP LTSIISAVVG
 661 ILLVVVLGVV FGILIKRRQQ KIRKYTMRRL LQETELVEPL TPSGAMPNQA QMRILKETEL
 721 RKVKVLGSGA FGTVYKGIWI PDGENVKIPV AIAVLRENTS PKANKEILDE AYVMAGVGSP
 781 YVSRLLGICL TSTVQLVTQL MPYGCLLDHV RENRGRLGSQ DLLNWCMQIA KGMSYLEDVR
 841 LVHRDLAARN VLVKSPNHVK ITDFGLARLL DIDETEYHAD GGKVPIKWMA LESILRRRFT
 901 HQSDVWSYGV TVWELMTFGA KPYDGIPARE IPDLLEKGER LPQPPICTID VYMIMVKCWM
 961 IDSECRPRFR ELVSEFSRMA RDPQRFVVIQ NEDLGPASPL DSTFYRSLLE DDDMGDLVDA
1021 EEYLVPQQGF FCPDPAPGAG GMVHHRHRSS STRSGGGDLT LGLEPSEEEA PRSPLAPSEG
1081 AGSDVFDGDL GMGAAKGLQS LPTHDPSPLQ RYSEDPTVPL PSETDGYVAP LTCSPQPEYV
1141 NQPDVRPQPP SPREGPLPAA RPAGATLERP KTLSPGKNGV VKDVFAFGGA VENPEYLTPQ
1201 GGAAPQPHPP PAFSPAFDNL YYWDQDPPER GAPPSTFKGT PTAENPEYLG LDVPV*
(SEQ ID NO:2)
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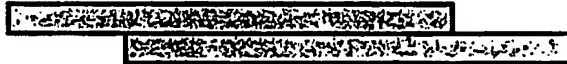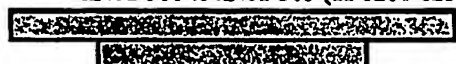Figure 2. Immunodominant T-cell epitopes in human HER2/neu protein.

		IFN-γ ELIspot Balb/c	IFN-γ Intracellular Balb/c	
			CD4[+]	CD8[+]
hNeu-1 to hNeu 30 (aa 1-131)	pool A	1,127		
hNeu-11 to hNeu-15 (aa 41-71)	subpool A_{III}	1,291		
hNeu-16 to hNeu-20 (aa 61-91)	subpool A_{IV}	1,057		
	hNeu15	1,095	0.27	46.35
	hNeu16	1,075	0.24	42.43
	hNeu15.1	518	0.15	25.67
	hNeu15.2	143	0.17	1.82
	hNeu15.3	1,258	0.22	48.78

57 Q G N L E L T Y L P T N A S L S F L Q
(SEQ ID NO: 5)

hNeu-31 to hNeu-60 (aa 121-251)	pool B	65		
hNeu-41 to hNeu-45 (aa 161-191)	subpool B_{III}	81		
	hNeu41	32	0.35	0.23
	hNeu42	42	0.47	0.23

161 L C Y Q D T I L W K D I F H K N N Q L
(SEQ ID NO: 6)

hNeu-301 to hNeu-311 (aa 1201-1255)	pool K	150		
hNeu-301 to hNeu-305 (aa 1201-1231)	subpool K_I	165		
	hNeu301	128	0.24	3.04
	hNeu301.1	13	0.10	0.06

1202 G G A A P Q P H P P P A F S P
(SEQ ID NO: 7)

	DMSO		0.10	0.12
	SEB		1.04	2.07

Figure 2.xls

FIGURE 3. Expression of HER2

A. HEK-293 Cells
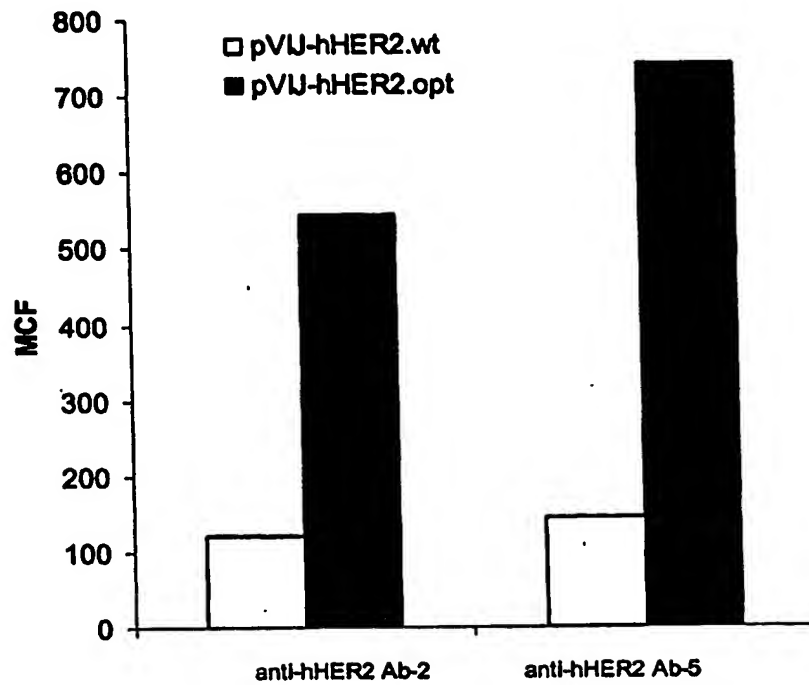
B. C2C7 Cells
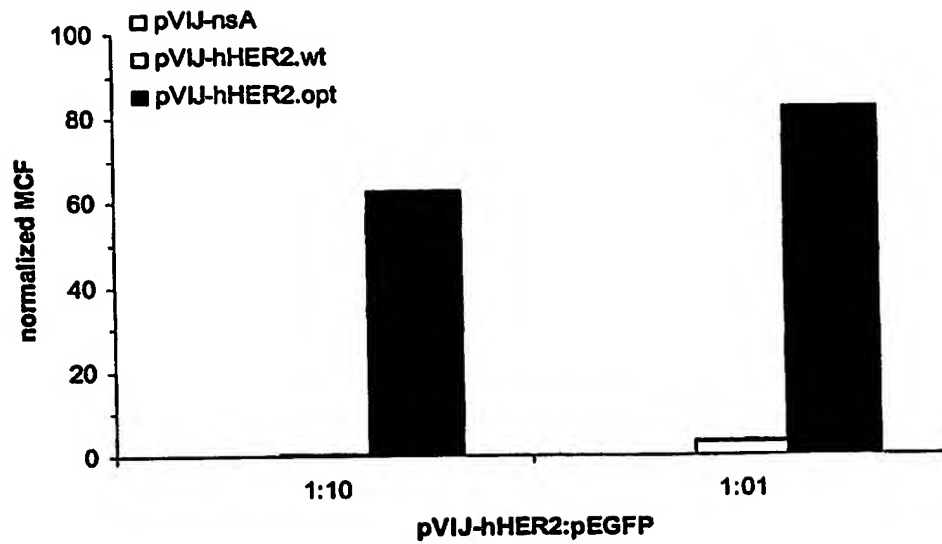
FIGURE 4. Immune Response to Human HER2

A

Mouse IFNγ ELISPOT

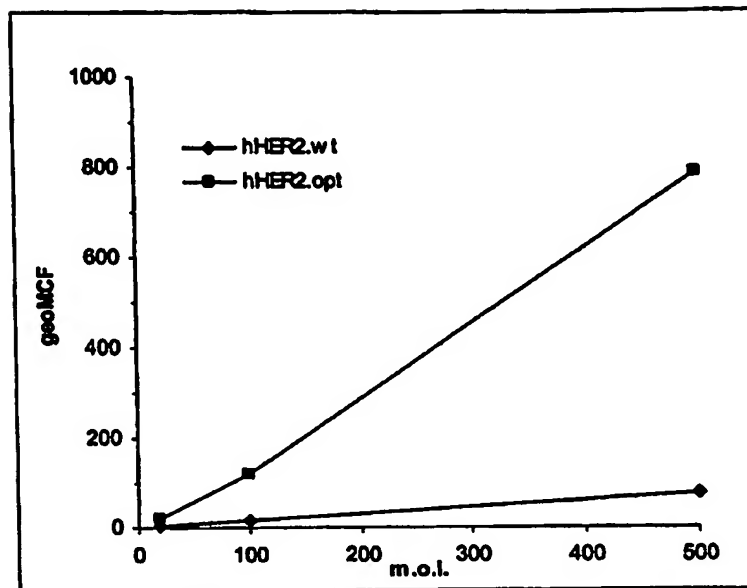|	mouse #	peptide	
		hNeu15.3 (CD8+)	hNeu42 (CD4+)
pV1J_hHER2.wt	14, 17	15	2
pV1J_hHER2.opt	1, 8	286	48
pV1J_hHER2wt	3, 7	28	16
pV1J_hHER2.opt	2, 5	250	60

B

Isotyping anti-humHER2 Ab

	IgG1	IgG2a
pV1J_hHER2.wt	<100	<100
pV1J_hHER2.opt	45,940	77,648

FIGURE 5. Immunization Regimens

	Ad5-hHER2		pV1J-hHER2 w/ ES	
	BALB/c	NeuT	BALB/c	NeuT
hNeu15.3	1,258	1,488	41	56
hNeu41	32	30	1	2
hNeu301	128	114	37	30

FIGURE 6. HER2 Expression in NMuLi Cells